CXXII. THE EXAMINATION OF IRRADIATED ZYMOSTEROL FOR THE PRESENCE OF VITAMIN D.

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(*Received June 29th, 1928.*)

It has recently been shown that ergosterol is associated in yeast with a highly unsaturated dextrorotatory sterol to which the name of zymosterol has been given [Smedley-MacLean, 1928]. This sterol appears closely to resemble ergosterol in its chemical properties; both contain three ethylenic linkages, both are readily susceptible to the oxidising action of air acting in the presence of light; they differ however markedly in their physical properties. Unlike ergosterol, a solution of zymosterol in alcohol produces no selective absorption bands in the ultra-violet region and, whereas ergosterol is strongly laevo-rotatory in ether or chloroform solution, zymosterol is dextrorotatory. Although the absence of selective absorption in the ultra-violet region rendered it unlikely that the new sterol could change on irradiation with ultra-violet light into a biologically active substance, it was desirable to establish this result by direct experiment.

The zymosterol was prepared from the mixture of sterols obtained on saponification of yeast fat and was separated from the ergosterol present in the mixture by fractional crystallisation. At present it has not been found possible to obtain a specimen of zymosterol entirely free from ergosterol; since both sterols are precipitated by digitonin, the amount of the impurity present was estimated by examination of the absorption spectrum in the ultra-violet region. The effect of a solution of one part of zymosterol in 5000 parts of alcohol was compared with that of solutions of different concentrations of ergosterol, and the amount of ergosterol present as impurity was taken as that present in the solution of pure ergosterol which produced the same absorption spectrum as the zymosterol solution. The spectroscopic examination showed that the specimen of zymosterol used in the first feeding experiment contained as much as 12 % of ergosterol. Subsequently a specimen was obtained in which the impurity was reduced to less than 5 %. The irradiation was carried out for 1 hour in 1 % ethereal solution at a distance of 32 cm. from a mercury vapour quartz lamp (Hewittic Company). The irradiated sterol was added to

an amount of hardened cotton-seed oil calculated so that a drop of the oil from a standardised pipette contained the minimum dose of irradiated sterol to be given.

Biological experiments.

For the biological tests, young rats were used and before a conclusive result was obtained four different diets had been employed. These were (1) a diet (*F* diet [see Hume, Smith and Smedley-MacLean, 1928]) deficient only in the fat-soluble vitamins A and D; (2) the same diet to which 20 % wheat germ had been added [see Leigh-Clare and Soames, 1928] to provide vitamin A; (3) diet 3143 of McCollum [McCollum *et al.*, 1921]; and (4) diet 84 of Sherman and Pappenheimer [1921]; the last two are both incomplete diets with a very low proportion of phosphorus. The percentage ash in the dried extracted bone and the degree of alkalinity of the faeces were examined in the rats fed on all the four diets. The method used for the examination of the faeces was a modification of that described by Jephcott and Bacharach [1926]; 0·2 g. faeces was rubbed up with 10 cc. distilled water and the hydrogen ion concentration determined by means of the capillator, bromothymol blue and phenol red being used as indicators. The results obtained in one series of readings were compared with those obtained by use of the hydrogen ion concentration cell and were found to be in close agreement. On the McCollum diet the degree of macroscopic rickets in the rib junctions was noted and on the Sherman-Pappenheimer diet an X-ray photograph of the right leg of each rat was taken as described by Rosenheim and Webster [1926].

Experiments using F diet.

Young rats of about 40 g. weight were placed upon the diet and after a period of from 4 to 8 weeks the weight curve became flat, showing that the reserve of vitamin D was exhausted; at this point the doses of irradiated sterol to be tested were administered daily for about 5 weeks. The rats retained a sufficient reserve of vitamin A to carry them through the experiment. Doses of 1/5,000, 1/10,000 and 1/20,000 mg. of irradiated ergosterol and zymosterol were used, and at the end of the experiment the percentage ash in the dried extracted leg bones was compared with that of untreated controls. As the zymosterol was never obtained free from ergosterol and might be owing all its activity to the impurity, the doses of irradiated sterols have been set out in Table I so as to show the dose of ergosterol given as impurity in each dose of zymosterol and these have been included in the series of ergosterol doses, each such dose appearing in the table under the dose of zymosterol of which it really formed a part. The doses of 1/5,000, 1/10,000 and 1/20,000 mg. of the first specimen of zymosterol used contained also doses of 1/40,000, 1/80,000 and 1/160,000 mg. of irradiated ergosterol respectively.

Reference to the table shows that the doses of 1/5,000 and 1/10,000 mg. of irradiated ergosterol and the same doses of zymosterol (containing 1/40,000

Table I. *Showing percentage of ash in dried extracted leg bones of rat.*

Irradiated zymosterol. Dose in thousandths of a milligram		0	—	—	—	1/5	1/10	—	1/20	—	—	1/100	1/200	1/400
Irradiated ergosterol. Dose in thousandths of a milligram		0	1/5	1/10	1/20	1/40	1/80	1/100	1/160	1/200	1/400	1/2000	1/4000	1/8000
						Percentage of ash.								
F diet	♂♀	47·1	57·1	57·3	—	56·7	55·9	—	—	—	—	—	—	—
	♀	49·6	—	—	—	—	—	—	—	—	—	—	—	—
	♂♀	52·6	54·6	56·8	—	53·6	53·9	—	—	—	—	—	—	—
	♀	53·3	—	—	—	—	—	—	—	—	—	—	—	—
	♂♀	—	—	—	56·2	—	—	—	50·8	—	—	—	—	—
	♀	48·8	—	—	—	—	—	—	—	—	—	—	—	—
F diet with 20 % wheat germ added	♂♀	55·3	—	—	—	—	—	55·9	—	—	—	55·2	—	—
	♀	—	—	—	—	—	—	—	—	>57·3*	—	>53·4*	54·6	—
	♂♀	46·2	—	—	—	—	—	—	—	56·0	—	51·0	52·7	—
	♀	49·6	—	—	—	—	—	60·3	—	54·7	—	54·8	51·7	—
McCollum diet	♂	40·6 Severe	—	—	—	—	—	—	—	—	40·1 Rather severe	—	—	39·0 Severe
	♀	—	—	—	—	—	—	49·2 Slight	—	—	—	42·9 Moderate	—	—
	♂	—	—	—	—	—	—	—	—	—	39·2 Moderate	—	—	30·1 Severe
	♀	—	—	—	—	—	—	45·2 Slight	—	—	—	37·7 Severe	—	—
	♂	—	—	—	—	—	—	46·9 Nearly normal	—	—	—	36·9 Severe	—	—
	♀	—	—	—	—	—	—	—	—	—	40·9 Moderate	—	—	35·9 Severe

* These ash determinations were calculated on the dried bone before it had been extracted and gave therefore too low values.

The terms "severe," "slight," etc. refer to the degree of rickets estimated by macroscopic inspection of rib junctions.

and 1/80,000 mg. ergosterol) all exercised a definite influence on calcification. Unfortunately only two animals were available for comparing the doses of 1/20,000 mg. ergosterol and 1/20,000 mg. zymosterol (containing 1/160,000 mg. ergosterol), but as far as such evidence goes it shows that the dose of 1/20,000 mg. ergosterol was effective but not the similar dose of zymosterol.

This simple comparison, however, suggested that the zymosterol was at least less effective than the ergosterol, and supposing that it was not effective at all indicated that the minimum dose of irradiated ergosterol which would exercise a definite effect upon calcification in rats receiving the *F* diet lay somewhere between 1/80,000 and 1/160,000 mg. Examination of the alkalinity of the faeces showed that, with rats fed on the *F* diet, there is not the same sharp differentiation between the controls and the rats receiving the doses of irradiated sterols as there is in rats fed on the less complete McCollum or Pappenheimer diet, although even on the *F* diet the faeces of the rats receiving irradiated sterol were on the whole more acid than those of the controls.

Experiments with F diet, containing 20 % *wheat germ.*

The experiment was begun as in the preceding instance on the *F* diet only, but the rats proved to have so large a reserve of vitamin D that by the time this was exhausted the reserve of vitamin A had also almost come to an end. In order to supply vitamin A, the diet containing 20 % wheat germ, which is a modification of the *F* diet, was therefore substituted after the rats had been 10 weeks on the *F* diet. Doses of 1/100,000 and 1/200,000 mg. irradiated ergosterol and the same doses of irradiated zymosterol (containing $\frac{1}{2\times10^6}$ and $\frac{1}{4\times10^6}$ mg. ergosterol) were then given daily for 42 days. At the end of that time the dried extracted bones were ashed and the percentage ash compared with that of controls. Reference to Table I shows that on this diet, complete except for the lack of vitamin D, the addition of even very small doses of irradiated sterol produced a marked effect. The effect of the dose of 1/100,000 mg. irradiated ergosterol is somewhat better and the dose of 1/200,000 mg. very definitely superior to that of the corresponding dose of irradiated zymosterol, but, though inferior, it is still not possible to say that the irradiated zymosterol has no activity of its own. The smallest dose of impurity is here one four-millionth milligram of irradiated ergosterol and, supposing the zymosterol to have no activity of its own, an effect on calcification would have to be ascribed to this extremely small dose, when added to this particular diet.

There was no sharp differentiation between the degree of alkalinity of the faeces of the controls and those of the rats receiving doses of the irradiated sterols. In most cases the p_H did not exceed 6·8.

Experiments with McCollum's diet 3143.

Since a response was obtained with such small doses of sterol on the wheat germ diet, making it almost impossible to find sharply the minimum dose, it

was thought that perhaps better differentiation would be shown on a diet of a more severe type. Diet 3143 of McCollum was therefore used after the rats had been for a fortnight on the *F* diet without wheat germ, it having been first intended to use that diet again. After 14 days on the *F* diet, the rats received the McCollum diet together with the dose to be tested over a period of 4–5 weeks.

Daily doses of 1/100,000 and 1/400,000 mg. of irradiated ergosterol were given and the same doses of irradiated zymosterol containing respectively $\frac{1}{2\times10^5}$ and $\frac{1}{8\times10^6}$ mg. ergosterol.

Macroscopic observations of the degree of rickets in the rib junctions were made and the percentage of ash in the dried extracted bone was also determined.

The results obtained in this experiment showed clearly that the only dose which produced any evidence of a definite protective action was the 1/100,000 mg. of ergosterol. The effect of the 1/400,000 mg. ergosterol was very slight but it seemed definitely to be somewhat better than that of either of the doses of zymosterol, the value of which appeared closely to resemble that of the controls.

With rats fed on the McCollum diet the inference could be drawn that the minimum dose of irradiated ergosterol which produces a significant effect is somewhere about 1/100,000 mg. and lies between this and 1/400,000 mg.

The faeces of all rats on this diet developed marked alkalinity (p_H 7·4 to 8·4); those of the rats receiving the 1/100,000 mg. of irradiated ergosterol were all less alkaline than those of the remaining animals, amongst which there appeared to be no significant differences.

Experiments with the Sherman-Pappenheimer diet.

It still remained uncertain whether zymosterol after irradiation had a certain value, though less than one-fourth that of an equal dose of irradiated ergosterol, or whether such activity as it acquired was due to the ergosterol present as impurity. An experiment was therefore carried out using the method of Rosenheim and Webster [1926] in which the rats are fed on the Sherman-Pappenheimer diet and an X-ray examination is made of the leg bones. These workers have examined a large number of rats with varying doses of ergosterol and have found that the usual minimum dose necessary to give a normal X-ray picture lies in the region of 1/10,000 mg. irradiated ergosterol [Rosenheim and Webster, 1927].

Young rats weighing 38 to 43 g. were used and arranged in four comparable groups, each containing six animals. One group was retained as controls, each animal of a second group received 1/5,000 mg. irradiated zymosterol, the animals of the third group were given 1/20,000 mg. irradiated ergosterol, while the rats in the last group were given 1/100,000 mg. irradiated ergosterol. Spectroscopic examination of the specimen of zymosterol used in this experiment had shown that it contained not more than 5 % ergosterol and therefore

a dose of 1/100,000 mg. of ergosterol corresponded to the maximum amount of this substance which could be present as impurity in the dose of zymosterol actually given. The animals received the diet and the daily dose for 25 days from the start of the experiment; they were then killed, the right leg dissected off and an X-ray picture taken. The result of this examination was conclusive (see Plate II); the controls gave a picture of severe rickets, the rats receiving 1/20,000 mg. ergosterol were almost normal while the bones of those receiving 1/5,000 mg. zymosterol (containing 5 % ergosterol as impurity) and 1/100,000 mg. ergosterol were indistinguishable. Any effect produced by the specimen of irradiated zymosterol used could therefore be ascribed to the effect of the ergosterol contained in it as impurity, and there was no evidence that the irradiated zymosterol was biologically active.

An estimation of the ash in the dried extracted bones gave the rather curious result that the rats receiving all three doses of the two sterols showed a definitely better calcification than the controls, but showed no differentiation among themselves. It seems possible therefore that on a diet such as the Sherman-Pappenheimer, very defective in phosphorus, a very small dose of vitamin D is sufficient to increase the percentage of ash in the bone but a much larger dose of this vitamin is not able further to increase this percentage in spite of the fact that the bone may give an almost normal appearance of calcification when the X-ray examination is made.

Discussion.

Besides establishing the fact that zymosterol is not capable of being activated by ultra-violet irradiation as ergosterol can be activated, the present series of experiments provides a basis of comparison for the minimum doses of irradiated ergosterol necessary to produce an effect when different diets are used and shows the results obtained by different methods of testing for vitamin D. When the more complete diets are given, the estimations of ash do not apparently differentiate sufficiently sharply between the effects of the various doses of the two sterols, or rather there is not sufficient difference shown over a large range of dosage. With diets deficient only in the fat-soluble vitamins, a dose of 2–8 millionths of a milligram of irradiated ergosterol appears to produce some effect on the percentage of ash in the bone, but increase of this dose produces very little more effect until a much larger dose is reached. At the same time in this series of experiments the inferiority of the irradiated zymosterol was definitely indicated.

The experiment using the McCollum diet in which only very small doses of the sterols were used gave a quite definite result; the X-ray test carried out on the rats fed on the Sherman-Pappenheimer diet was certainly the most conclusive. In judging between the methods it must, however, be remembered that the last-named method was used in the final experiment when the results of the previous experiments were available, and that this method having been extensively made use of by Rosenheim and Webster their wide experience as

to dosage was available when the experiment was planned. In testing for the presence of vitamin D the use of the McCollum or Sherman-Pappenheimer diet appears to give more sharply differentiated results and to have the advantage of being more quickly carried out. With these severe diets, the minute doses which appear to have a definite effect in increasing the percentage of ash in the bone produce no result. With the McCollum diet a marked effect was produced by a dose of 1/100,000 mg. of the irradiated ergosterol though this dose was insufficient to ensure complete protection. Using the Sherman-Pappenheimer diet, our experience agreed with that of Rosenheim and Webster that a dose of rather more than 1/20,000 mg. was necessary to give a normal picture.

The results of the examination of the faeces were in general agreement with the other methods of testing above described when the Sherman-Pappenheimer or McCollum diet was used, but were not sufficiently sharply differentiated when the more complete diet was given.

We wish to express our thanks to Dr Rosenheim and Mr Webster for their help in arranging for the X-ray examinations of the bones and to Dr N. S. Lucas for carrying out for us the spectroscopic examinations necessary to determine the percentage of the ergosterol present in our specimens of zymosterol. We desire also to acknowledge our indebtedness to the Medical Research Council and to the Department of Scientific and Industrial Research for grants which have enabled us to carry out this investigation.

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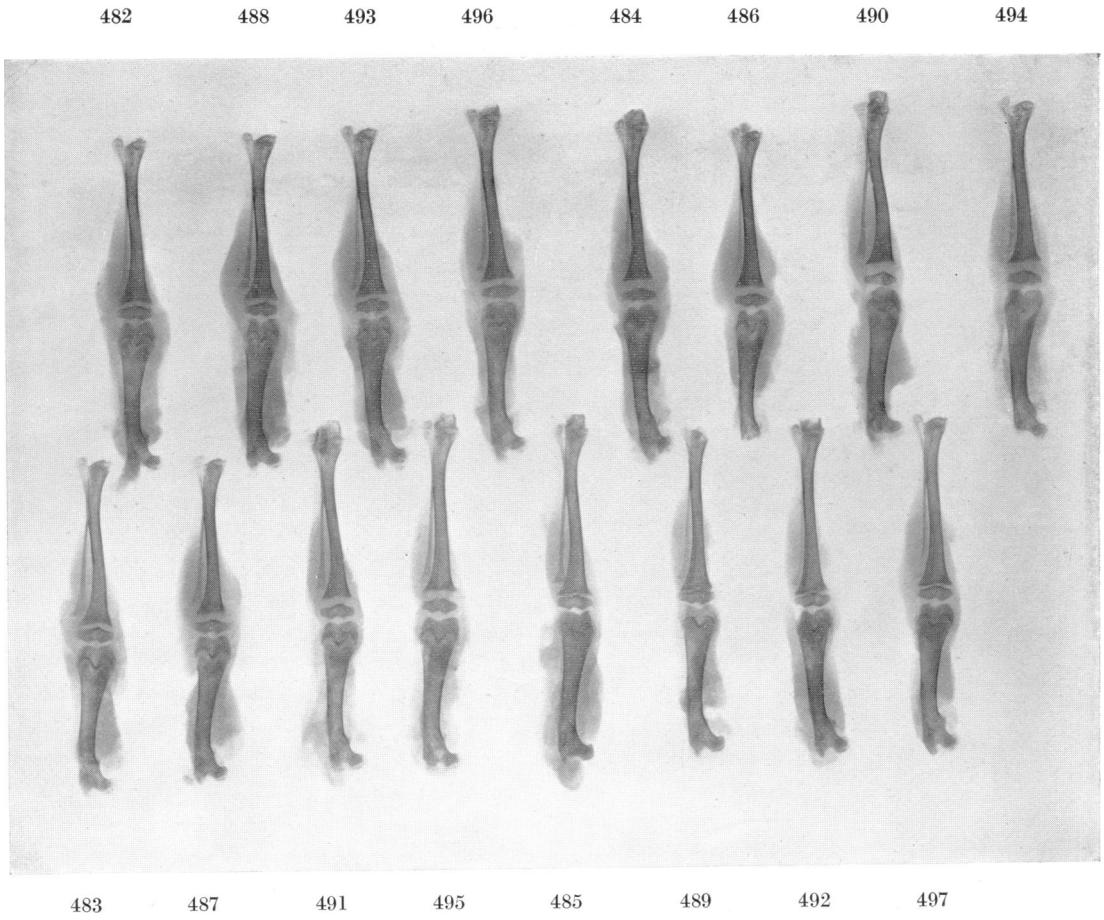

Results of X-ray examination of leg bones of rats on Sherman-Pappenheimer diet.

497, 492, 489 and 485. Rats received 1/20,000 mg. irradiated ergosterol daily.
495, 491, 487 and 483. Rats received 1/5,000 mg. irradiated zymosterol.
482, 488, 493 and 496. Rats received 1/100,000 mg. irradiated ergosterol.
484, 486, 490 and 494. Controls.